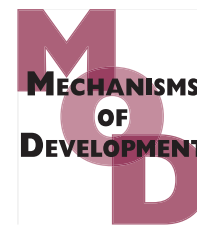


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# Hes1 and Hes5 regulate vascular remodeling and arterial specification of endothelial cells in brain vascular development

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## ABSTRACT

The vascular system is the first organ to form in the developing mammalian embryo. The Notch signaling pathway is an evolutionarily conserved signaling mechanism essential for proper embryonic development in almost all vertebrate organs. The analysis of targeted mouse mutants has demonstrated essential roles of the Notch signaling pathway in embryonic vascular development. However, Notch signaling-deficient mice have so far not been examined in detail in the head region. The bHLH genes *Hes1* and *Hes5* are essential effectors for Notch signaling, which regulate the maintenance of progenitor cells and the timing of their differentiation in various tissues and organs. Here, we report that endothelial-specific *Hes1* and *Hes5* mutant embryos exhibited defective vascular remodeling in the brain. In addition, arterial identity of endothelial cells was partially lost in the brain of these mutant mice. These data suggest that *Hes1* and *Hes5* regulate vascular remodeling and arterial fate specification of endothelial cells in the development of the brain. *Hes1* and *Hes5* represent critical transducers of Notch signals in brain vascular development.

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## 1. Introduction

The vascular system is one of the first organs to form in the developing mammalian embryo. During the early stages of vascular development, endothelial cell (EC) precursors differentiate and coalesce into a primitive network of undifferentiated blood vessels in the process of vasculogenesis. In the later stages of vascular development, this primary vascular plexus is remodeled into a network of large and small vessels by the process of angiogenesis (Carmeliet, 2000; Risau, 1997). This process is controlled by various signaling mole-

cules and their downstream pathways. These pathways include vascular endothelial growth factor (VEGF) and its receptors, transforming growth factor- $\beta$  (TGF- $\beta$ ) and its receptors, angiopoietin 1 and its receptor Tie2, and ephrin-B ligands and EphB receptors (Carmeliet et al., 1996; Suri et al., 1996; Wang et al., 1998).

The Notch signaling pathway is an evolutionarily conserved signaling mechanism essential for proper embryonic development in almost all vertebrate organs. Mammals express four Notch receptors (Notch1–4) and five ligands [Jagged (Jag) 1 and 2, and Delta-like (Dll) 1, 3 and 4] (Gridley, 2007;

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Kageyama et al., 2005, 2007). Notch is a transmembrane protein and activated by its ligands, Dll or Jag proteins. Upon activation, the intracellular domain (ICD) of Notch is cleaved off and transferred into the nucleus, where the Notch ICD forms a complex with the DNA-binding protein RBP-J. This complex induces expression of *Hes* genes (Kageyama et al., 2008a,b). The analysis of targeted mouse mutants has demonstrated roles of the Notch signaling pathway in embryonic vascular development. In mice, the absence of Notch signaling results in a similar phenotype characterized by defective vascular remodeling in the extraembryonic yolk sac, placenta, and embryo proper (Alva and Iruela-Arispe, 2004; Gridley, 2007). These embryos also exhibited a loss of expression of arterial markers in arterial vessels. Notch signaling regulates the specification of arterial fate in ECs during mouse embryogenesis (Alva and Iruela-Arispe, 2004; Gridley, 2007).

The bHLH genes *Hes1* and *Hes5* are essential effectors for Notch signaling, which regulate the maintenance of progenitor cells and the timing of their differentiation in various tissues and organs (Hatakeyama et al., 2004, 2006; Hojo et al., 2008, 2000; Ohtsuka et al., 1999). These features of *Hes* genes have been reported especially in the development of the nervous system. However, only a few *in vitro* studies have shown a role of *Hes* genes as a downstream effector of Notch in ECs. *In vitro* studies using human arterial ECs have supported that *Hes1* is a downstream effector of Notch1 in the vasculature similarly as in other tissues (Albig et al., 2008; Liu et al., 2006; Quillard et al., 2008). In humans and mice, the Notch3-*Hes5* signaling pathway is crucial for the development of pulmonary arterial hypertension (Li et al., 2009). However, no study using *Hes* mutant mice has so far shown a role of *Hes* genes in vascular development.

The bHLH genes *Hesr* (*Hey*, *HRT*, *gridlock*, or *CHF*) belong to a related but different subclass from *Hes* genes (Fischer and Gessler, 2003). The knockout (KO) of *Hesr2* revealed a critical function during heart development with ventricle septum defect, persistent foramen ovale, tricuspid valve stenosis, and cardiomyopathy as the predominant anomalies. However, *Hesr2* KO mice did not show vascular defects, in contrast to mutants of zebrafish *gridlock*, a homolog of *Hesr2*, which exhibit coarctation of the aorta (Fischer et al., 2004; Kokubo et al., 2005). In addition, *Hesr1* KO mice showed no obvious phenotype. However, mouse embryos lacking both *Hesr1* and *Hesr2* are embryonic lethal due to cardiovascular malformations, and have a single large ventricle and arterial differentiation defect, suggesting that *Hesr1* and *Hesr2* are required in mediating Notch signaling in the developing cardiac and vascular systems (Fischer et al., 2004; Kokubo et al., 2005).

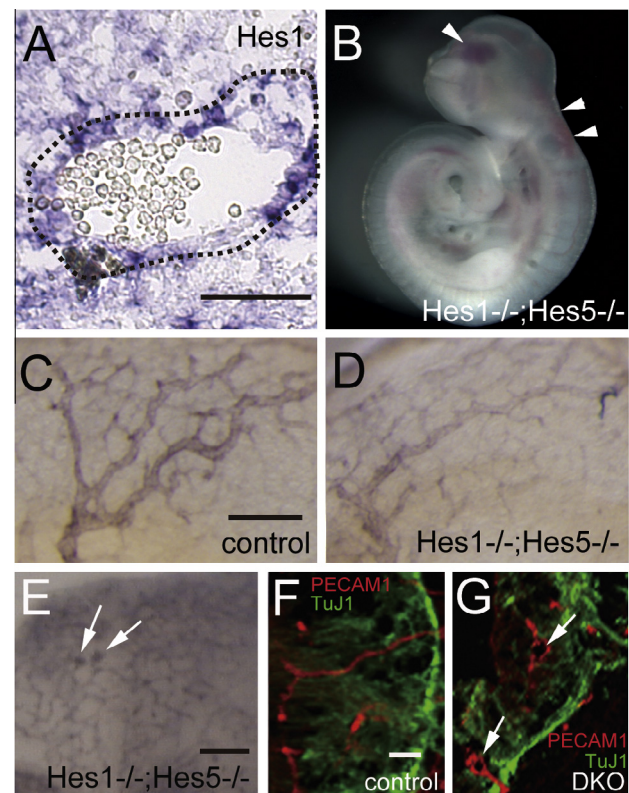
Although mutations in human *Notch3* cause CADASIL, a hereditary vascular dementia, *Notch3* KO mice develop normally (Joutel et al., 1996; Krebs et al., 2003). In adult *Notch3* KO mice, cerebral arteries are enlarged and have thinner smooth muscle cell (SMC) coat (Domenga et al., 2004). Although it is expected that the Notch pathway have an important role in vascular development of the brain, Notch signaling-deficient mice have so far not been examined in detail in the head region. Here, we show that *Hes1* and *Hes5* regulate vascular remodeling of the brain during embryogenesis by analyzing *Hes*-mutant mouse embryos. We also show that

*Hes1* and *Hes5* play a role in establishing arterial cell fate and identity of ECs of cerebral blood vessels.

## 2. Results

### 2.1. *Hes*-mutant embryos exhibit abnormal brain vascular development

We first examined *Hes1* expression in the internal carotid artery of mouse embryos by *in situ* hybridization (ISH). *Hes1* was expressed in almost all ECs of the developing internal



**Fig. 1 – Vascular defects in the brain of *Hes1*; *Hes5* DKO mice.** (A) ISH of *Hes1*. *Hes1* was expressed in the internal carotid artery (dashed line) at E10.5. (B) *Hes1*; *Hes5* DKO embryos showed hemorrhage in the brain and the spinal cord at E10.5 (arrowheads). (C–E) Whole-mount PECAM-1-stained control (C) and *Hes1*; *Hes5* DKO (D and E) embryos at E10.5. The surface of the mesencephalon is shown. In *Hes1*; *Hes5* DKO mice, large major branches of the internal carotid artery were decreased in number, and instead, small branches were increased (D and E) compared with the control (C). Moreover, in double-mutant mice, abnormal microvascular networks and vascular malformations (abnormal angiomas, indicated by arrows) were also observed (E). (F and G) Immunostaining for PECAM-1 (red) and Tuj1 (green) on sections through the forebrain of E10.5 control (F) and *Hes1*; *Hes5* DKO (G) embryos. Small fragmented vessels (arrows) were observed in the neural tube of *Hes1*; *Hes5* DKO embryos (G) compared with the control (F). Bars, 50  $\mu$ m (A, F and G); 200  $\mu$ m (C and D); 100  $\mu$ m (E).

carotid artery at E10.5 (Fig. 1A), suggesting that *Hes1* plays a role in brain vascular development. To reveal the role of *Hes* genes in brain vascular development, we decided to examine *Hes*-mutant mice. Because it has been reported that *Hes5* compensates *Hes1* during the development of various organs, we examined *Hes5* expression in the internal carotid artery by ISH, but *Hes5* was not detectable in ECs of the developing internal carotid artery in both wild type and *Hes1* KO embryos. However, it has been reported that *Hes5*, which was not detected in ECs of the aorta, was highly induced after genetic deletion of *Hes1* (Guju et al., 2013), we examined both *Hes1* KO and *Hes1*; *Hes5* double knockout (DKO) embryos to understand the role of *Hes* genes in cerebral vascular development (Hatakeyama et al., 2004, 2006; Kita et al., 2007; Ohtsuka et al., 1999). In almost half of *Hes1* KO (data not shown) and *Hes1*; *Hes5* DKO (Fig. 1B) embryos, hemorrhage was observed in the cerebrospinal nervous system, suggesting that *Hes1* and *Hes5* may play a role in brain vascular development.

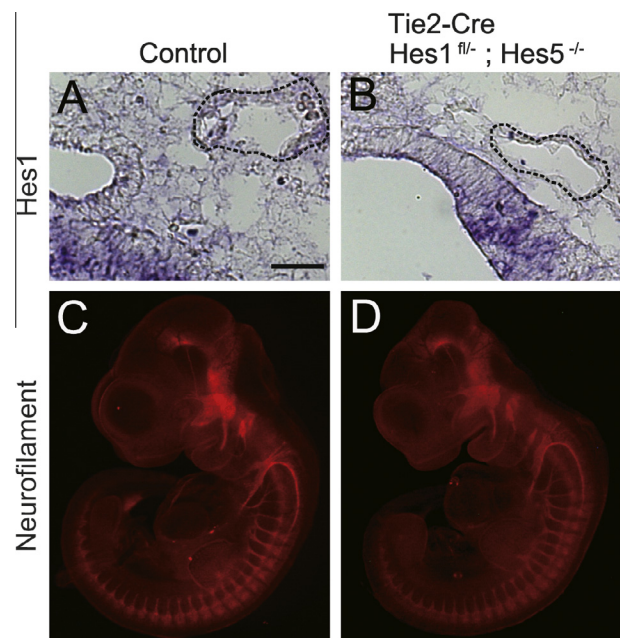
To investigate the patterning of the vasculature in *Hes*-mutant embryos, we performed whole-mount immunostaining for the endothelial marker platelet-endothelial cell adhesion molecule (PECAM)-1. In E10.5 *Hes1* KO embryos, abnormal microvascular networks were observed over the brain compared with the control, but this phenotype was not obvious probably owing to compensation by *Hes5* (data not shown). In *Hes5* KO embryos, there was no phenotype in vascular development (data not shown). Interestingly, in E10.5 *Hes1*; *Hes5* DKO embryos, the number of large branches of the internal carotid artery was decreased, and instead, the number of small branches was increased compared with the control (Fig. 1C and D). Abnormal microvascular networks over the brain were also observed in *Hes1*; *Hes5* DKO mice (Fig. 1E). In *Hes1*; *Hes5* DKO embryos, vascular malformations (abnormal angiomas) were also observed in the brain (Fig. 1E, arrows). Next, in order to examine these abnormal microvascular networks in more detail, we performed immunostaining for PECAM-1 and Tuj1 on sections through the forebrain of E10.5 *Hes1*; *Hes5* DKO embryos. In *Hes1*; *Hes5* DKO embryos, many small fragmented vessels were observed in the neural tube (Fig. 1G, arrows) compared with the control (Fig. 1F). Because it takes time for the formation of secondary vascular malformations due to cerebral hemorrhage, it is considered unlikely that brain hemorrhage leads to vascular malformations in mouse embryos. Therefore, these abnormal vessels and angiomas may be the cause of hemorrhage in the nervous system of *Hes* mutant embryos. These phenotypes can be explained by defective remodeling of the primary vascular plexus of the brain. These data suggest that *Hes1* and *Hes5* regulate vascular remodeling in brain vascular development during embryogenesis of mice.

## 2.2. Generation of conditional *Hes1*; *Hes5* double knockout mice

It has previously been shown that the size, shape and cyto-architecture of the central nervous system are severely disorganized in *Hes1*; *Hes5* DKO mice, because neural stem cells are prematurely depleted without giving rise to later-born cell types such as astrocytes and ependymal cells (Hatakeyama et al., 2004). Therefore, we cannot dismiss the possibility that

this disruption of neural tube structures may account for the phenotypes of cerebral blood vessels in *Hes1*; *Hes5* DKO embryos. To exclude this possibility, we generated endothelial-specific *Hes1* conditional knockout (cKO) mice by crossing *Hes1* floxed mice and TIE2Cre mice (Imayoshi et al., 2008; Koni et al., 2001). The TIE2 gene encodes an angiopoietin receptor, a member of the receptor tyrosine kinase family, which is expressed in ECs throughout development (Koni et al., 2001). Because *Hes5* KO mice are apparently normal, we generated *Hes1* cKO mice on a *Hes5*-null background (*Hes1*; *Hes5* cKO mice).

To examine the Cre recombinase activity in the developing ECs of TIE2Cre mice, we crossed these mice with ROSA26 reporter mice (Soriano, 1999). Cre recombinase activity was detected in ECs of blood vessels in the whole body including the head (data not shown). To show the evidence of *Hes1* ablation in ECs of *Hes1*; *Hes5* cKO mice, ISH of *Hes1* was performed. No *Hes1* expression was detected in ECs of the internal carotid artery, but *Hes1* was expressed normally in the neural tube in *Hes1*; *Hes5* cKO embryos (Fig. 2A and B). Next, to show that the nervous system develops normally in *Hes1*; *Hes5* cKO mice, we performed whole-mount immunostaining for the neuronal marker neurofilament. No phenotype was detected in the developing nervous system of *Hes1*; *Hes5* cKO embryos (Fig. 2C and D). Therefore, we decided to analyze *Hes1*; *Hes5* cKO mice in order to investigate the roles of *Hes1* and *Hes5* in brain vascular development.



**Fig. 2 – Generation of *Hes1*; *Hes5* cKO mice. (A and B) Direct evidence of ablation of *Hes1* in the internal carotid artery by Tie2-Cre transgene. ISH of *Hes1*. No *Hes1* expression was detected in ECs of the internal carotid artery (dashed lines) of E10.5 *Hes1*; *Hes5* cKO embryos (B) compared with the control (A). *Hes1* expression was observed in the neural tube of both mutant and control mice. (C and D) Whole-mount immunostaining for the neuronal marker neurofilament. No apparent phenotype was detected in the developing nervous system of *Hes1*; *Hes5* cKO mice (D) compared with the control mice (C) at E10.5. Bar, 50  $\mu$ m.**

### 2.3. *Hes1* and *Hes5* regulate vascular remodeling of the brain during embryogenesis

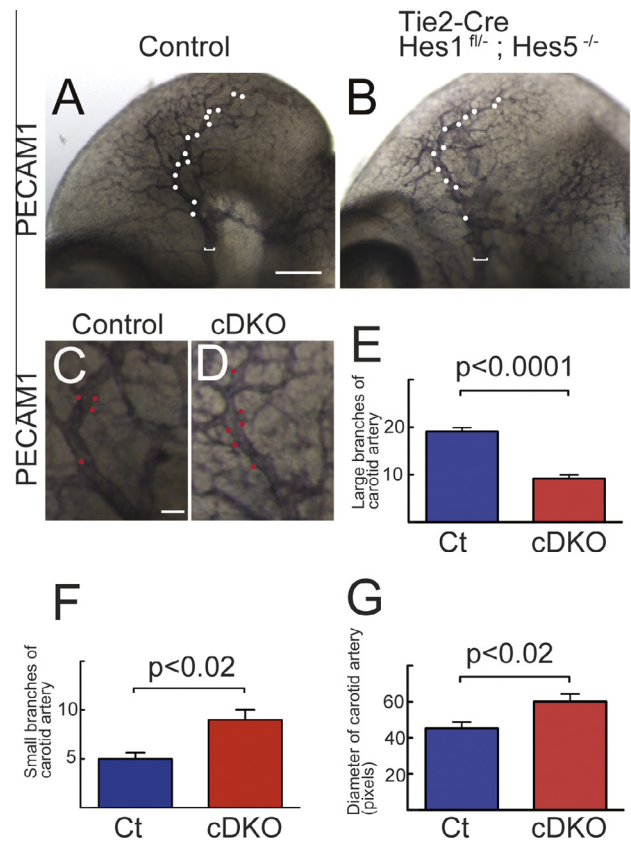
To investigate the patterning of the cerebral vasculature in *Hes1*; *Hes5* cKO embryos, we performed whole-mount immunostaining for PECAM-1 (Fig. 3A–D). In E10.5 *Hes1*; *Hes5* cKO mice, the number of large branches of the internal carotid artery was significantly decreased, and instead, the number of small branches was significantly increased compared with the control (Fig. 3A–F). The term “large branches” means arterial branches, which are more than one-half the diameter of the parent artery, and the term “small branches” means arterial branches, which are less than one-half the diameter of the parent artery. Abnormal microvascular networks were also observed over the brain of *Hes1*; *Hes5* cKO embryos. These results recapitulated the vascular phenotype of global *Hes1*; *Hes5* DKO embryos, and showed that vascular remodeling was affected in the brain of *Hes1*; *Hes5* cKO embryos. In addition, in *Hes1*; *Hes5* cKO mice, the internal carotid artery had a significantly increased diameter compared with the control (Fig. 3A, B and G), and the mechanism of this phenotype will be discussed in the next Section (2.4).

Next, we examined cross sections of *Hes1*; *Hes5* cKO embryos stained for PECAM-1 in order to investigate the abnormal microvascular network of the brain in more detail. In *Hes1*; *Hes5* cKO mice, many small fragmented vessels were observed in the neural tube compared with the control (Fig. 4A and B). Although not statistically significant, the number of fragmented vessels tended to increase in *Hes1*; *Hes5* cKO embryos compared with the control (Fig. 4C). In addition, PECAM-1 positive area of fragmented vessels was significantly decreased in *Hes1*; *Hes5* cKO embryos compared with the control (Fig. 4D). These findings are consistent with the abnormal microvascular network in the brain of *Hes1*; *Hes5* cKO embryos. Taken together, these data suggest that *Hes1* and *Hes5* regulate vascular remodeling in the brain development.

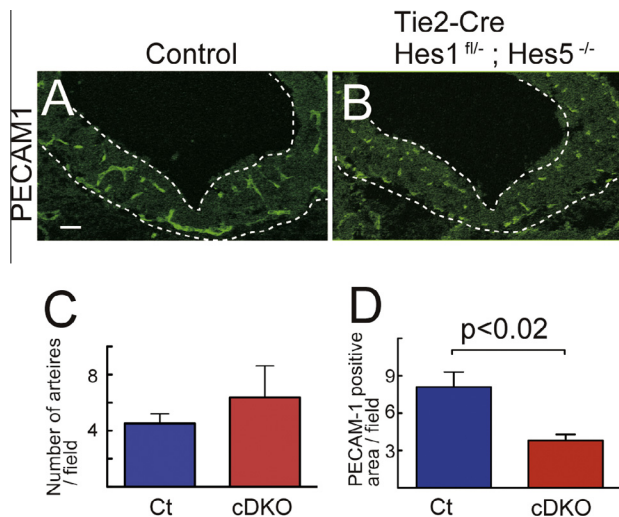
### 2.4. *Hes1* and *Hes5* regulate arterial specification of ECs of brain blood vessels

Previous studies in zebrafish and mice have shown that Notch signaling is essential for arterial versus venous cell fate decisions. Therefore, we examined the expression of CD44, an arterial endothelial marker, in E10.5 *Hes1*; *Hes5* cKO embryos to assess vascular lineage and identity (Fig. 4A and B) (Fischer et al., 2004; Krebs et al., 2004). Internal carotid arteries were positive for CD44 both in *Hes1*; *Hes5* cKO mice and the control (Fig. 5A and B, arrows). However, CD44 positive ECs were not detected in blood vessels of the neural tube of *Hes1*; *Hes5* cKO embryos (Fig. 5B), although CD44 positive ECs were faintly detected in the neural tube of the control (Fig. 5A, arrowhead). This suggests that *Hes1* and *Hes5* control the arterial cell fate specification of ECs in the head region.

The maturation of blood vessels requires the formation of vascular smooth muscle cells (vSMCs) around the nascent endothelial tube. This process occurs earlier in arteries than in veins, and is regulated by signaling from ECs to the surrounding differentiating vSMCs (Carmeliet, 2000; Winkler et al., 2011). Therefore, we examined the expression of  $\alpha$



**Fig. 3 – Vascular defects in the brain of *Hes1*; *Hes5* cKO mice.** (A–D) Whole-mount PECAM-1-stained control (A and C) and *Hes1*; *Hes5* cKO (B and D) embryos at E10.5. In *Hes1*; *Hes5* cKO embryos, large branches of the internal carotid artery were decreased in number (A and B, white dots indicate branch points of large branches), and instead, small branches were increased compared with the control (C and D, red dots indicate branch points of small branches). The term “large branches” means arterial branches, which are more than one-half the diameter of the parent artery, and the term “small branches” means arterial branches, which are less than one-half the diameter of the parent artery. Abnormal microvascular networks were also observed in the brain of *Hes1*; *Hes5* cKO embryos. Furthermore, in *Hes1*; *Hes5* cKO mice (B), the diameter of the internal carotid artery (brackets) was larger than in the control mice (A). (E) Quantification of branch points of large branches. Ten cerebral hemispheres obtained from 5 independent embryos were examined for each genotype. Large major arterial branches were significantly decreased in *Hes1*; *Hes5* cKO mice compared with the control ( $p < 0.0001$ , t-test). (F) Quantification of branch points of small branches. Ten cerebral hemispheres obtained from 5 independent embryos were examined for each genotype. Small arterial branches were significantly increased in *Hes1*; *Hes5* cKO mice compared with the control ( $p < 0.02$ , t-test). (G) Quantification of the diameter of the internal carotid artery. Ten internal carotid arteries obtained from 5 independent embryos were examined for each genotype. The diameter of the internal carotid artery was significantly increased in *Hes1*; *Hes5* cKO mice compared with the control ( $p < 0.02$ , t-test). Bars, 200  $\mu$ m (A–D).



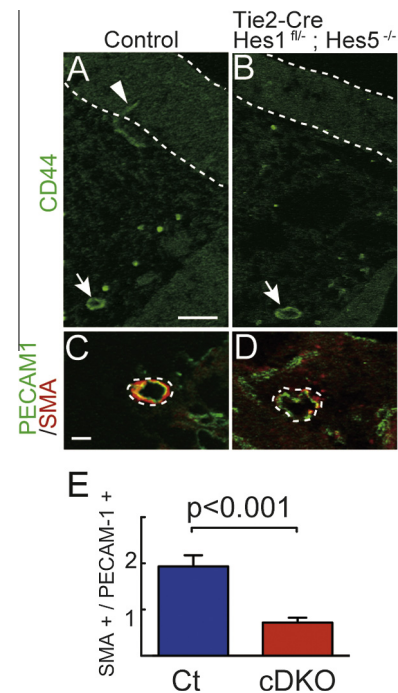
**Fig. 4 – Vascular defects in the brain of *Hes1*; *Hes5* cKO mice.** (A and B) Immunostaining for PECAM-1 on sections through the hindbrain of E10.5 control (A) and *Hes1*; *Hes5* cKO (B) embryos. Many small fragmented vessels were observed in the neural tube of *Hes1*; *Hes5* cKO embryos compared with the control. (C) Quantification of the number of fragmented arteries in the neural tube. Three independent embryos were examined for each genotype. Although not statistically significant, the number of fragmented arteries tended to increase in *Hes1*; *Hes5* cKO mice compared with the control. (D) Quantification of PECAM-1 positive area of fragmented vessels in the neural tube. The PECAM-1 positive area was significantly decreased in *Hes1*; *Hes5* cKO mice compared with the control ( $p < 0.02$ , t-test). Bars, 100  $\mu$ m (A and B).

smooth muscle actin ( $\alpha$ SMA), the earliest marker of vSMCs, in the internal carotid artery (Fig. 5C and D) (Chang et al., 2012; Domenga et al., 2004; Kokubo et al., 2005; Krebs et al., 2004). The expression level of  $\alpha$ SMA was significantly reduced in the internal carotid artery of *Hes1*; *Hes5* cKO embryos (Fig. 5C–E) compared with the control, whereas PECAM-1 expression appeared unaffected. The layer of vSMCs was affected probably due to the defective signaling from ECs in *Hes1*; *Hes5* cKO embryos. In addition, in *Hes1*; *Hes5* cKO mice, the diameter of the internal carotid artery was larger than in the control mice (Fig. 3A, B and G). This abnormal layer of vSMCs may be the cause of dilatation of the internal carotid artery in *Hes1*; *Hes5* cKO embryos (Domenga et al., 2004). Taken together, these results show that ECs of cerebral blood vessels of *Hes1*; *Hes5* cKO embryos have partially lost arterial identity, and that *Hes1* and *Hes5* play a role in establishing arterial cell fate and identity of ECs during the development of the brain.

### 3. Discussion

#### 3.1. *Hes1* and *Hes5* regulate vascular remodeling in the brain during embryogenesis

Previous reports have shown that *Notch1*-mutant embryos exhibited deficits in angiogenic remodeling (Gridley, 2007; Krebs et al., 2000). Although deletion of *Notch4* did not affect



**Fig. 5 – Defective arterial specification in the brain of *Hes1*; *Hes5* cKO embryos.** (A and B) Immunostaining for CD44 on sections through the hindbrain of E10.5 control (A) and *Hes1*; *Hes5* cKO (B) embryos. Internal carotid arteries were positive for CD44 both in *Hes1*; *Hes5* cKO mice and the control (arrows). However, CD44 positive ECs were not detected in the neural tube of *Hes1*; *Hes5* cKO mice (B), although CD44 positive ECs were faintly detected in the neural tube of the control (A, arrowhead). Three independent embryos were examined for each genotype. (C and D) Immunostaining for  $\alpha$ SMA (red) and PECAM-1 (green) on sections of E10.5 control (C) and *Hes1*; *Hes5* cKO (D) embryos. The expression of  $\alpha$ SMA was down-regulated in the internal carotid artery of *Hes1*; *Hes5* cKO embryos (D) compared with the control (C), whereas PECAM-1 expression was not affected. (E) Ratio of  $\alpha$ SMA positive area to PECAM-1 positive area of the internal carotid artery. This ratio was significantly decreased in *Hes1*; *Hes5* cKO mice compared with the control ( $p < 0.02$ , t-test). Four independent embryos were examined for each genotype. Bars, 100  $\mu$ m (A and B); 50  $\mu$ m (C and D).

vascular development, *Notch1*; *Notch4* DKO embryos displayed more severe phenotypes than embryos deficient in *Notch1* alone (Krebs et al., 2000). Global deletion of *Notch1* resulted in defective embryonic morphogenesis, including defects in vascular development, neurogenesis, and somitogenesis. Unexpectedly, endothelial-specific *Notch1* cKO embryos displayed severe defects not only in vascular remodeling but also in tissues distinct from the vasculature (Limbourg et al., 2005). Specifically, the forebrain neural tube in endothelial-specific *Notch1* cKO mice was grossly degenerated. Therefore, vascular phenotypes of the brain of *Notch1* mutant embryos could not be analyzed. Heterozygous deletion of *Dll4* showed increased numbers of smaller arterial branches of the internal carotid artery, although the major arteries such as the internal carotid artery appeared normal (Gale et al., 2004; Suchting et al.,

2007). In endothelial-specific *Jag1* cKO embryos, the blood vessels in the head region were less finely branched (High et al., 2008; Xue et al., 1999). In *Hes1*; *Hes2* DKO embryos, large cranial vessels appeared truncated. However, the possibility that the defective vessel branching was secondary to the disruption of the neural tube cannot be dismissed because *Hes1*; *Hes2* DKO embryos were strongly reduced in overall size (Fischer et al., 2004; Kokubo et al., 2005). Unfortunately, this phenotype was not investigated in endothelial-specific *Hes1* mutant embryos (Xin et al., 2007). Thus, the mechanism of brain vascular development has not been elucidated so far by analyzing Notch signaling-deficient mice.

In this study, we generated and analyzed endothelial-specific *Hes1*; *Hes5* cKO mice. In *Hes1*; *Hes5* cKO mice, neural structures were not affected and developed normally. In *Hes1*; *Hes5* cKO mice, the number of large branches of the internal carotid artery was decreased, and instead, the number of small branches was increased. Abnormal microvascular network over the brain was also observed in *Hes1*; *Hes5* cKO embryos. These findings show that vascular remodeling was affected in the brain of *Hes1*; *Hes5* cKO embryos. Our data suggest that *Hes1* and *Hes5* represent critical transducers of Notch signals in brain vascular development.

### 3.2. *Hes1* and *Hes5* modulate arterial fate decision of ECs in brain vascular development

In *Notch1* KO or *Hes1*; *Hes2* DKO embryos, CD44 expression was down-regulated in the dorsal aorta, suggesting that the arterial cell fate specification of ECs is controlled by *Notch1* or a combination of *Hes1* and *Hes2* (Fischer et al., 2004). Loss of arterial markers in *Notch1* KO or *Hes1*; *Hes2* DKO embryos occurs specifically in the dorsal aorta. Although endothelial-specific RBPJ cKO embryos also exhibited the same phenotype, brain blood vessels were not examined in this mutant embryo (Krebs et al., 2004). In our study, CD44-positive ECs were decreased in the neural tube of *Hes1*; *Hes5* cKO embryos, although the internal carotid artery was positive for CD44 both in *Hes1*; *Hes5* cKO mice and the control. This result suggests that *Hes1* and *Hes5* play a role in establishing arterial cell fate or identity in brain vascular development.

In *Hes1*; *Hes5* cKO embryos, *Hes1* was ablated only in ECs, but not in adjacent vSMCs. However, the expression level of  $\alpha$ SMA was reduced around ECs of the internal carotid artery. This result shows that endothelial-specific deletion of *Hes1* and *Hes5* resulted in a marked reduction of vSMCs, suggesting that *Hes1*; *Hes5* double-null ECs were defective in signaling, such as platelet derived growth factor (PDGF)-B, TGF- $\beta$ , and angiopoietin to adjacent SMC precursors to promote their differentiation (Winkler et al., 2011). It has been reported that endothelial loss of RBPJ resulted in down-regulation of TGF- $\beta$ 2, and that knockdown of *Notch1* inhibited cyclic strain-induced angiopoietin1 expression in ECs (Copeland et al., 2011; Morrow et al., 2007). These previous reports may support our idea described above. This also suggests that ECs of brain blood vessels of *Hes1*; *Hes5* cKO embryos have partially lost arterial identity, and that *Hes1* and *Hes5* play a role in

establishing arterial cell fate and identity of ECs in brain vascular development. By contrast, in the endothelial-specific *Jag1* cKO embryos, the expression of SMC markers was severely diminished, although Notch signaling in ECs remained intact and the arterial specification of ECs was also normal in the absence of *Jag1* (High et al., 2008). The primary role of endothelial *Jag1* is to potentiate the development of neighboring vSMCs. Endothelial *Jag1* is considered to signal directly to Notch receptors on adjacent vSMCs. Thus, *Hes1* and *Jag1* may be involved in the development of vSMCs in a completely different mechanism, although it is not possible to confirm this idea, because we could not examine *Jag1* in *Hes1*; *Hes5* cKO mice.

Taken together, our data suggest that *Hes1* and *Hes5* control the arterial cell fate specification of EC precursors in brain vascular development.

### 3.3. Possible roles of *Hes1* and *Hes5* in cerebrovascular disease

Arteriovenous malformations (AVMs) of the central nervous system are congenital disorders resulting from aberrant differentiation of the mesoderm during embryonic development, leading to lesions that bear morphological resemblance to the early anastomotic plexuses formed during central nervous system vascular embryogenesis (Hashimoto et al., 2007; Takagi et al., 2006). Although intracranial AVMs are recognized as a cause of stroke, the mechanism of their development and enlargement is still unclear. In mice, *Dll4*<sup>+/-</sup>, *Notch1* KO and endothelial-specific RBPJ cKO embryos exhibited AVMs in the body, which were caused by fusion of the dorsal aorta and the common cardinal vein (Krebs et al., 2004, 2010). However, brain AVMs were not detected in these mutant mice. In our study, *Hes1*; *Hes5* DKO embryos exhibited vascular malformations in the brain, although we did not show the direct shunt flow in these lesions. Interestingly, mouse embryos with conditional activation of the *Notch1* gene in ECs also exhibited AVMs in the body (Krebs et al., 2010). The inducible expression of an activated *Notch4* transgene in adult mice results in vessel arterialization, and causes AVMs in several organs, including liver, uterus, skin and brain (Carlson et al., 2005; Murphy et al., 2008). In induced brain AVMs of these transgenic mice, *Hes1* signal was increased in ECs (Murphy et al., 2009). In human brain AVMs, the analysis of surgical specimens has shown that the expression of *Hes1* and *Notch1* was increased (ZhuGe et al., 2009). Both the loss of function and gain of function *Notch1* mutant embryos had AVMs in the body. Formation of AVMs during embryonic development is likely due to an inability of the vascular beds to maintain distinct arterial and venous identities (Krebs et al., 2010). Therefore, it is considered that *Hes1* expression in human brain AVMs and mouse induced AVMs is consistent with the phenotype of *Hes1*; *Hes5* cKO mice. Taken together, *Hes1* and *Hes5* may play a role in the formation of vascular malformations in the brain.

In summary, *Hes1* and *Hes5* control angiogenic remodeling and the arterial cell fate specification of EC precursors in brain vascular development. *Hes1* and *Hes5* represent critical transducers of Notch signals in brain vascular development.

In addition, *Hes1* and *Hes5* may play a role in the formation of brain vascular malformations.

## 4. Experimental procedures

### 4.1. Mice

TIE2Cre mice were a gift from Pandelakis A. Koni (Koni et al., 2001). *Hes1*<sup>−/−</sup>; *Hes5*<sup>−/−</sup>, *Hes1*<sup>fl/fl</sup>; *Hes5*<sup>−/−</sup> and *Tie2*<sup>+/-Cre</sup>; *Hes1*<sup>+/-</sup>; *Hes5*<sup>−/−</sup> mice were obtained by crossbreeding (Imayoshi et al., 2008; Kita et al., 2007). *Hes* genes were deleted in ECs by crossing *Tie2*<sup>+/-Cre</sup>; *Hes1*<sup>+/-</sup>; *Hes5*<sup>−/−</sup> mice with *Hes1*<sup>fl/fl</sup>; *Hes5*<sup>−/−</sup> mice (*Hes1*; *Hes5* cKO mice). Genotypes of TIE2Cre, *Hes1* and *Hes5* mutant, and *Hes1* floxed mice were determined as previously described (Cau et al., 2000; Hirata et al., 2001; Kita et al., 2007; Koni et al., 2001). All analyses were performed between littermates. *Hes1*<sup>+/-</sup>; *Hes5*<sup>−/−</sup> mice were found to have no significant phenotype in the developing vascular system and were therefore used as controls. For analysis of Cre recombinase activity, TIE2Cre mice were crossed with ROSA26 reporter mice (a gift from P. Soriano) and analyzed by X-gal staining as previously described (Imayoshi et al., 2010; Soriano, 1999).

### 4.2. In situ hybridization

Digoxigenin-labeled antisense RNA probes corresponding to fragments of *Hes1* cDNAs were synthesized *in vitro*. *In situ* hybridization was performed as previously described (Hirata et al., 2001; Hojo et al., 2000; Kita et al., 2007). Briefly, 16-μm-thick cryosections were treated with proteinase K, refixed with 0.2% glutaraldehyde and 4% paraformaldehyde, washed with 0.1% Tween 20 in PBS, and hybridized with RNA probe in 50% formamide, 5× SSC, 1% SDS, 50 μg/ml heparin, and 50 μg/ml tRNA solution at 65 °C overnight. After hybridization, sections were washed at 65 °C in 50% formamide, 5× SSC, 1% SDS, treated with ribonuclease, washed in 50% formamide, and 2× SSC, washed in Tris-buffered saline, and incubated with alkaline-phosphatase-conjugated antibody against digoxigenin at 4 °C overnight. After incubation, the sections were washed with 0.1% Tween 20 in Tris-buffered saline three times, and in 100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl<sub>2</sub>, and 0.1% Tween 20 solution once. For a color development reaction, 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used as substrates. Labeled preparations were imaged using a Carl Zeiss Axiophoto microscope equipped with a CCD camera.

### 4.3. Whole-mount immunostaining

Embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4 °C for 3 h and were bleached with 0.1% H<sub>2</sub>O<sub>2</sub> at 4 °C for 16 h. Then, the embryos were incubated in anti-PECAM or anti-neurofilament antibody solutions [antibody was diluted at 1/2 in TS-PBS (1% Triton X-100; 10% FBS in PBS)] at 4 °C for 3 days and next with peroxidase (Chemicon) or Alexa 594 (Molecular Probes) – conjugated secondary antibodies overnight at 4 °C. The peroxidase deposits were visualized by 4-chloro-1-naphthol (Hatakeyama et al., 2006).

### 4.4. Immunostaining

Immunostaining was performed with the following antibodies: rat anti-PECAM (BD Pharmingen), mouse anti-αSMA (Sigma), mouse anti-Tuj1 (Covance), and rat anti-CD44 (BD Pharmingen) antibodies. Cryosections were incubated in 5% normal goat serum and 0.1% Triton X-100 at room temperature for 1 h, then incubated with primary antibodies at 4 °C overnight (Hojo et al., 2000; Kita et al., 2007). Donkey or goat anti-species IgG conjugated with Alexa 488 or Alexa 598 (Molecular Probes) was used for a secondary antibody. For all antibodies, no significant signal was detected in negative controls. Sections were analyzed with LSM510 confocal microscopy (Carl Zeiss).

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